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14. ABSTRACT

A protective secretion produced by epidermal mucus cells in stingrays is being investigated to understand its role in wound healing and to identify mucus-associated antimicrobial compounds with the potential for development into novel therapeutics to treat wound infection pathogens. Fresh mucus from two species of ray (cownose ray, *Rhinoptera bonasus*, and Atlantic stingray, *Dasyatis sabina*) contains proteins in an aqueous supernatant and a viscous pellet. The magnesium salt of trifluoroacetic acid was successful in obtaining mucus pellet extracts enriched in low MW compounds demonstrating low but measurable antibiotic activity. Mucus pellets also contain symbiotic bacteria, many of which demonstrate antibiotic activity. 135 bacterial isolates cultured from cownose ray and 11 from Atlantic stingray epidermal mucus demonstrated antibiotic activity against at least one human pathogenic tester strain in primary screens performed at Mote Marine Laboratory. Of the 11 Atlantic stingray isolates, 6 demonstrated antibiotic activity against pathogenic bacterial tester strains screened at University of South Florida Center for Biological Defense. Culturable libraries of all isolates have been cryopreserved. Experimental wounding studies resulted in wounds that healed without infection or inflammation. A consistent observation is the formation of raised fibrous tissue in the center of the wounds within three weeks, which gradually dissipates to uniform scar tissue across the wound. Preliminary histology on healed wounds provided valuable baseline data on restructuring of epidermal and dermal tissue during the healing process.

15. SUBJECT TERMS
stingray epidermal mucus, antimicrobial activity, wound healing, wound infection pathogens, bacterial symbionts, culturable bacterial isolates

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INTRODUCTION:

Combat wounds, particularly blast wounds, are highly susceptible to infection and delayed healing. As a result, U.S. military caregivers are constantly seeking new antibiotics to treat soldiers wounded in combat arenas as well as those recovering in hospitals. Animal models of rapid wound healing can provide significant insights into novel approaches of treating combat wounds. There are numerous examples of remarkable wound healing in sharks and their skate and ray relatives in which traumatic wounds heal completely and quickly. Our hypothesis is that rapid and infection-free wound healing in elasmobranch fishes is related to antimicrobial activity present in epidermal mucus and that mucus-derived compounds will be effective against a variety of wound infection pathogens. The aims of this project are to identify antimicrobial activity against wound infection pathogens by epidermal mucus secretions of stingrays and skates, to determine the contribution of mucus to the rate of healing and resistance to infection in these fish, to establish biochemical profiles for mucus protein/peptide factors and to isolate antimicrobial compounds extracted from epidermal mucus. The studies to achieve these aims are coordinated among personnel at four institutions: Mote Marine Laboratory, Daemen College Center for Wound Healing Research, University of South Florida Center for Biological Defense, and Clemson University Animal & Veterinary Sciences. The anticipated research findings will impact the CMDRP Basic Research Program **Polytrauma and Blast Injury** project tasks directed toward **Wound Infection Prevention & Management** and **Antimicrobial Countermeasures** by identifying antibiotic compounds with the potential for development into novel antimicrobial agents that will facilitate the treatment of polymicrobial infections of combat-related wounds.

BODY:

Research during Year 2 of this three-year funded project contributed information toward portions of most Tasks described in the SOW. Many of the Task objectives are planned to be continuous or on-going throughout the project. As a result, none of the Tasks has been completed. Since many of the Subtask activities built upon data acquired during the initial year's studies, some activities are further along than others.

Research accomplishments associated with Tasks and Subtasks outlined in the proposed Statement of Work are described below.

Task 1. Collect animals and epidermal mucus

Subtasks 1a & 1b. Animal Care and Use Review Office (ACURO) and Mote Marine Laboratory IACUC review and approval. Protocols received approval from both the USAMRMC ACURO. Because IACUC approvals at Mote Marine Laboratory are renewed annually, documentation of Mote's IACUC renewal was submitted to ACURO and received continued approval on 17 September, 2012.

Subtask 1c. Animal containment facility (MML)
Animal containment facilities prepared during Year 1 for the controlled experimental portions of the study are functioning well, with costs budgeted for routine maintenance and animal care being utilized. Experimental tanks utilize temperature-controlled recirculating natural seawater maintained with biological and particle filtration and 12 hr on, 12 hr off photoperiods.

Subtask 1d. Collect animal specimens (MML)
Collection of research animals is an ongoing activity during the project. Cownose rays, *Rhinoptera bonasus*, and Atlantic stingrays, *Dasyatis sabina*, are collected passively, by surrounding them in shallow water with a seine net, transferring them with dip nets to an onboard live-well, and either sampling them and immediately releasing them unharmed (cownose rays) or transporting them to Mote Marine Laboratory (Atlantic stingrays). Cownose rays are an active, schooling ray that require large holding tanks and are not ideal for long-term captive studies. Because of their relative size and local abundance, however, they can be sampled at time of capture and released unharmed. Atlantic stingrays are smaller than cownose rays, but because of their sedentary and solitary behavior, they are ideal for long-term captive maintenance in smaller tanks compared to those required for cownose rays, and can be easily manipulated for experimental procedures. Consequently, Atlantic stingrays were used in experimental wounding studies conducted during Year 2.

Subtask 1e. Collect epidermal mucus from non-wounded and experimentally wounded animals (MML).
Methods to collect epidermal mucus have been developed during Year 1 continue to be successful and consist of sampling individual rays by passive scraping of the pectoral fin surfaces with a sterile scoopula and transferring the mucus to sterile culture tubes.

Task 2. Determine antimicrobial activity of epidermal mucus

Subtask 2a. Prepare epidermal mucus extracts
Year 2 experiments with mucus and mucus extracts included 1) studies of the stability of mucus components by assessing the effects of storage temperature and time, as well as freeze-thaw cycles on protein composition of stingray mucus extracts, 2) preliminary efforts to extract proteins separated on electrophoretic gels, and 3) numerous extraction procedures to develop optimal methods to isolate compounds from aqueous supernatant and pellet fractions of freshly collected mucus in preparation for bioactivity screening and characterization of proteomic profiles.

<u>Protein Stability Studies – Effects of storage temperature and time</u>

To determine the optimal storage conditions for epidermal mucus for protein analysis, a pilot study was conducted to assess protein composition and concentrations in mucus supernatant stored at two temperatures (4℃ and -80℃) over 16 days. On day 0, mucus was collected from *D. sabina*, pooled and centrifuged to separate supernatant and pellet fractions, distributed into aliquots, and stored either at 4℃ or -80℃. Protein composition and concentrations were assessed on day 0 and again on days 8 and 16. Frozen aliquots remained at temperature for the duration of the experiment and were not subjected to freeze thaw cycles. Separate aliquots subjected to freeze-thaw cycles (data not shown) exhibited similar results to temperature stable aliquots. Protein composition was assessed using SDS polyacrylamide gel electrophoresis and protein concentrations assessed using the Bradford assay. Little change in protein banding patterns on electrophoretic gels was also observed as a function of storage conditions (Figure 1). Overall, changes in protein concentration (Table 1) at either storage temperature were minor, with slight increases likely attributed to evaporation.

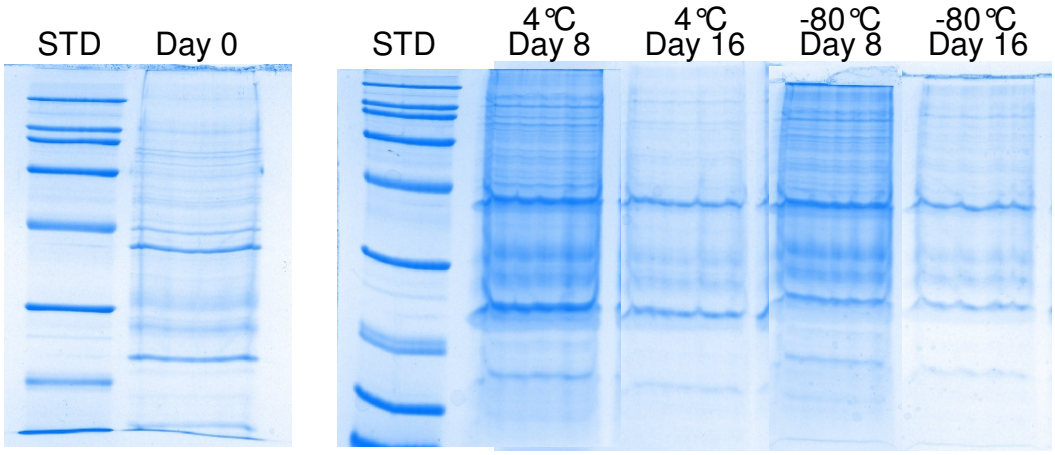

Figure 1. Protein composition of *D. sabina* mucus supernatant stored at 4℃ and -80℃. Day 0, 12% gel; Days 8 and 16, 15% gels.

Table 1. Protein concentration of *D. sabina* mucus supernatant stored at 4℃ and -80℃.

	Protein Concentration (mg/mL)	
Timepoint	4ºC	-80ºC
Day 0	2.98	2.98
Day 8	2.95	3.09
Day 16	3.48	3.31

Protein Stability Studies – Effects of freeze-thaw cycles

Stability of mucus proteins subjected to repetitive freezing and thawing was tested by collaborators at Daemen College. Mucus samples frozen immediately after collection were shipped overnight to Daemen College, where they were thawed, divided into three portions and frozen at -80℃. The three portions were frozen and thawed twice, five times, or eight times, and protein compositions were analyzed using SDS-PAGE. As seen in Figure 2, protein banding patterns did not change with freeze-thaw cycles.

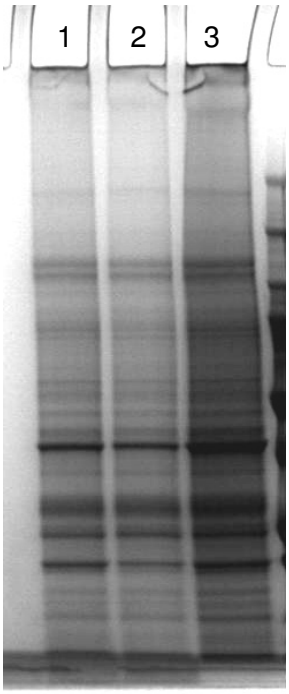

Figure 2. Banding patterns of mucus proteins subjected to two (lane 1), five (lane 2), and eight (lane 3) freeze-thaw cycles. SDS-PAGE on 4-12% gradient gels.

Although mucus proteins appear to be quite stable to duration and temperature of storage as well as repetitive freezing and thawing, it is recommended that to ensure sample integrity, the time between mucus collection and analysis will be minimized. Long-term storage will be at -80℃ and samples will not be subjected to unnecessary freeze-thaw events.

Extraction of Proteins Separated on Electrophoretic Gels. Procedures utilized at Mote Marine Laboratory to extract proteins from stingray mucus using published methods for acidic and organic extractions, followed by bioactivity assay, have been minimally successful to date due to interference in bioactivity assays from residual extraction reagents. Direct extraction of mucus proteins from polyacrylamide gels (both SDS and native) following electrophoretic separation is being explored as an alternative method for isolating protein components for testing of bioactivity.

Initial attempts to recover proteins from SDS-PAGE gels have been successful. Pooled mucus from *D. sabina* was centrifuged at 10,000 x g to separate supernatant and pellet fractions. Mucus supernatant was separated in duplicate lanes of 12% SDS-PAGE gels. Using prestained molecular weight standards as a guide, an unstained lane containing supernatant was excised and divided into 3 fractions. The fractions were placed in vials containing PBS. Proteins were allowed to elute passively out of the gel overnight at 4°C. Gel slices were removed and eluted proteins were lyophilized. Protein fractions were reconstituted in 500 µL PBS, protein content determined by the Bradford assay, and subjected to SDS-PAGE to evaluate protein recovery.

Results from the Bradford assay indicated protein was recovered at measurable concentrations from gel fractions 2 and 3 (data not shown). Although bands are faint, gel electrophoresis confirmed the recovery of several protein bands, especially the major band in fraction 2 (Figure 3).

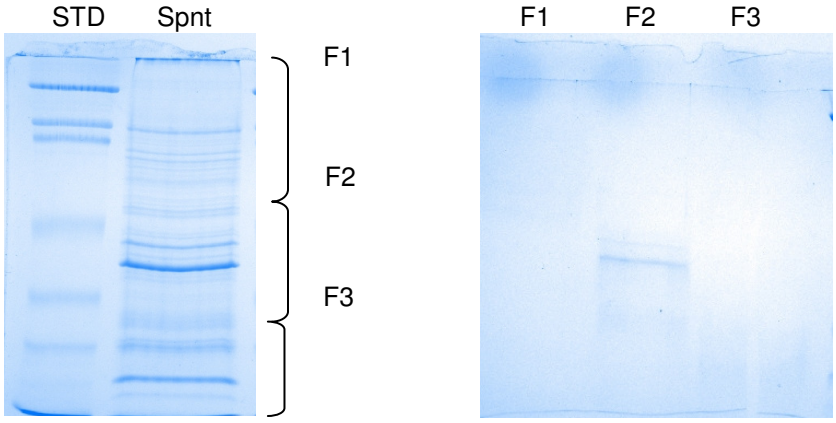

Figure 3. Recovery of protein from excised fractions of 12% SDS-PAGE gel. A) Protein standards and mucus supernatant (Spnt). B) Extracted fractions (F1, F2 and F3).

Work to optimize recovery of protein from SDS-PAGE electrophoresis is on-going with plans to screen gel-extracted fractions for bioactivity. Extraction of native PAGE gels, in which proteins are separated under non-denaturing conditions, is also being explored.

Mucus Extraction: Extraction with Detergents and Chelating Agents. At Daemen College, samples were extracted with one of three treatments: 0.1% Tween 80, 1% lithium dodecyl sulfate (LDS), or 1.5 mM EDTA:1 mM DTT, diluted in a Tris buffered saline (10 mM Tris and 150 mM NaCl, pH 7.4) and separated into aqueous supernatant and mucus pellet fractions. Mucus pellets were washed with the extraction solution, rocked gently while on ice and sampled at 1 hour and again at 1.5 hours. New wash solution was added to each pellet and sampled after 1 hour. For all treatments, the initial wash removed many proteins while subsequent washes resulted in concentrating two low molecular weight bands also present in the initial wash (Figure 4). Banding profiles from Tween-80 treated and LDS treated mucus samples were similar. EDTA-DTT extracted mucus contained many of the same bands as the detergent-extracted mucus, but also included some additional bands that were solubilized.

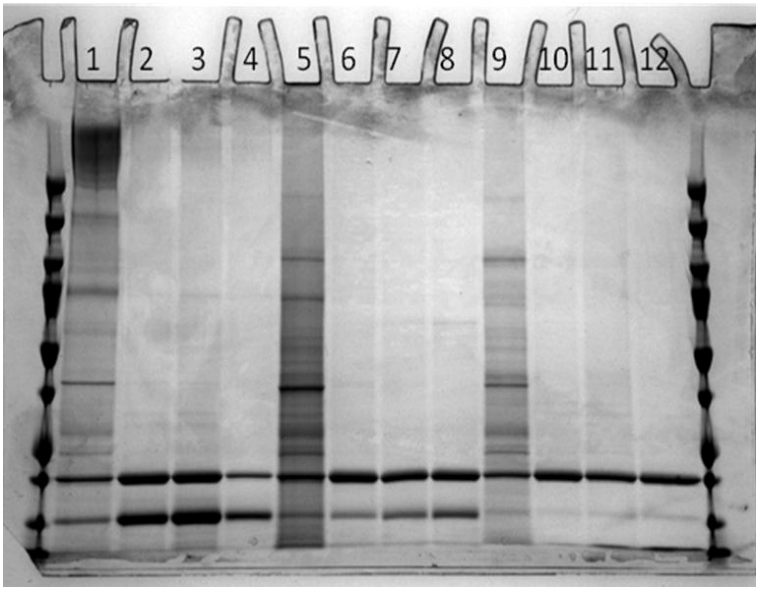

Figure 4. Banding patterns of mucus proteins extracted with EDTA-DTT (lanes 1-4), lithium dodecyl sulfate (lanes 5-8) and Tween-80 (lanes 9-12). Of the four lanes per treatment, the first lane represents proteins extracted from aqueous supernatant, while the second and third lanes represent proteins extracted from mucus pellets washed with the extraction solution and sampled at 1 hour and 1.5 hours. The fourth lane represents proteins extracted from mucus pellets extracted an additional time and sampled after 1 hour. SDS-PAGE on 4-12% gradient gels.

Mucus Extraction: Extraction with Enzymes

Collaborators at Daemen College also tested papain and pepsin for their abilities to liberate proteins from the mucus pellet. Papain is a cysteine protease and a common active ingredient in meat tenderizers. It is often used to dissociate cells in the first step of cell culture preparations. Pepsin is an enzyme released by the chief cells in the stomach that degrades food proteins into peptides.

Both enzymes liberated protein but did not completely liquefy the mucus pellet. Banding patterns were inconsistent between samples and both methods present problems associated removal of residual enzyme from the mixture of extracted proteins (Figure 5).

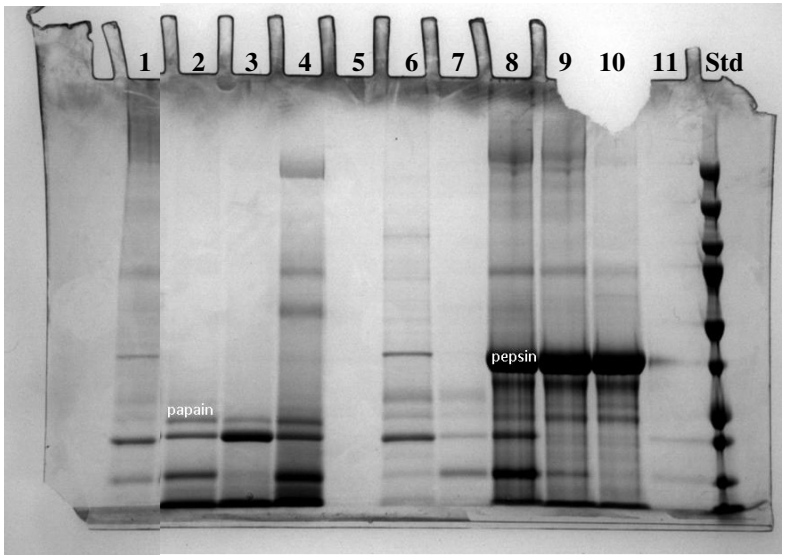

Figure 5. SDS polyacrylamide 4-12% gradient gels of mucus pellet proteins following various treatments with Papain and Pepsin. Lane 1: EDTA-DTT extract; Lane 2-4: Mucus pellet + Papain for 30, 40, and 60 min; Lane 5: Mucus pellet Papain buffer control, 30 min, 37 ℃; Lane 6: Tris-NaCl extract; Lane 7: Mucus pellet Pepsin buffer control, 30 min, 37deg; Lanes 8-10: Mucus pellet + Pepsin for 30, 40, and 60 min; Lane 11: Mucus pellet Pepsin buffer control, 60 min, 37 ℃.

Mucus Extraction: Mechanical Dispersement

An IKA T10 disperser and 10G generator were used at Daemen College in an attempt to liquefy intact mucus mechanically. A mucus pellet of reduced size remained after centrifugation to separate aqueous supernatant from the remaining mucus pellet. The gel in Figure 6 shows that protein bands are similar among preparations.

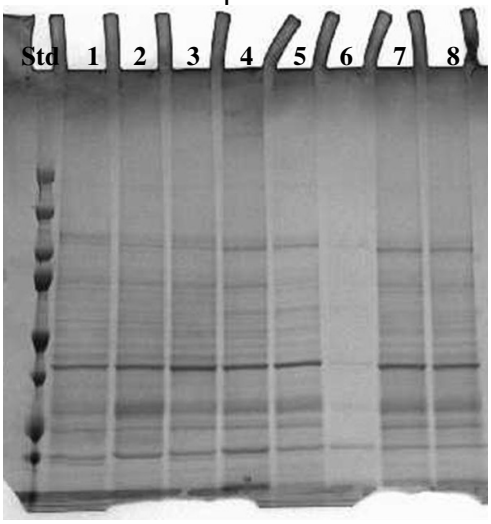

Figure 6. SDS polyacrylamide 4-12% gradient gels of aqueous supernatant and mucus pellet proteins following mechanical disruption. Lanes 1-5: mucus pellet; Lane 6: probe wash solution; Lane 7-8: aqueous supernatant.

Mucus Extraction: Extraction with Magnesium Trifluoroacetate

Many of the procedures commonly used to extract compounds from biological tissue involve organic chemicals or acids. Not only are these reagents harsh, but residual amounts of these reagents remain with the extracted mucus material and have interfered with the spectroscopic turbidity assay being developed to assess antibiotic activity. One promising reagent for mucus extraction is the magnesium salt of trifluoroacetic acid, one of the acids commonly used in extractions of biological tissues. The reagent is prepared by reacting magnesium oxide with trifluoroacetic acid, resulting in a neutral salt with a pH between 7 and 8.

Fresh samples of aqueous supernatant, mucus pellet and seawater were extracted using equal volumes of 1 M $Mg(TFA)_2$ in 15mL polypropylene centrifuge tube and placed on orbital mixer for 2 hrs at 4° C. Samples were subjected to centrifugation to remove residual material and/or undissolved pellet. The mucus pellet was extracted a second time and supernatants were combined. Resulting supernatants were dialyzed (3,500 MW cutoff) against 0.05 M ammonium bicarbonate using and lyophilized.

SDS polyacrylamide gel electrophoresis of protein components from two different batches of Atlantic stingray mucus extracted with $Mg(TFA)_2$ are shown in Figure 7. The protein pattern reveals that not only does the extract have many of the proteins present prior to extraction, but some of the lower molecular weight components appear to be enriched in the mucus pellet extract.

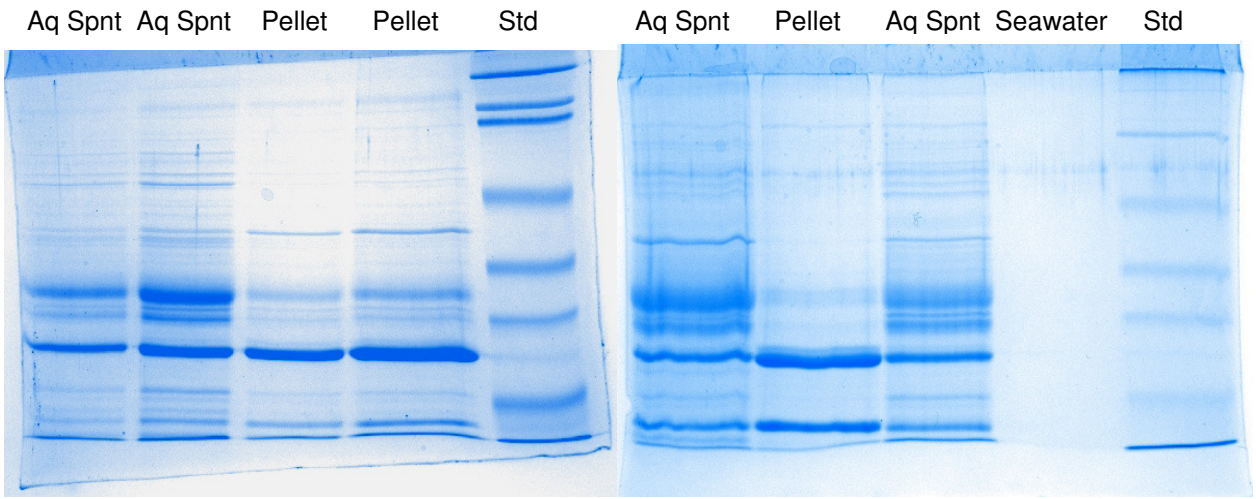

Figure 7. SDS polyacrylamide gel (12% gel) of proteins/subunits extracted from aqueous supernatant (Aq Spnt) and mucus pellet with magnesium trifluoroacetate, $Mg(TFA)_2$.

Subtask 2b. Conduct primary antibiotic screens of epidermal mucus for antimicrobial activity

Antibiotic Activity of Magnesium Trifluoroacetate-extracted Mucus

$Mg(TFA)_2$-extracted samples were screened for antibiotic activity using the tester pathogen, *Bacillus subtilis*. *B. subtilis* was grown overnight and a working stock of mid-log phase culture was prepared. With the absorbance at 600 nm of control cultures defined as "no activity" and a Penicillin/Streptomycin mix as an inhibitory control (100% inhibition), the extent to which mucus extracts inhibit growth of the pathogen cultures are determined spectrophotometrically as a change in turbidity. The turbidity change is converted to a percent inhibition with the absorbance of the control culture defined as 0% inhibition. Results from this assay are shown in Figure 8.

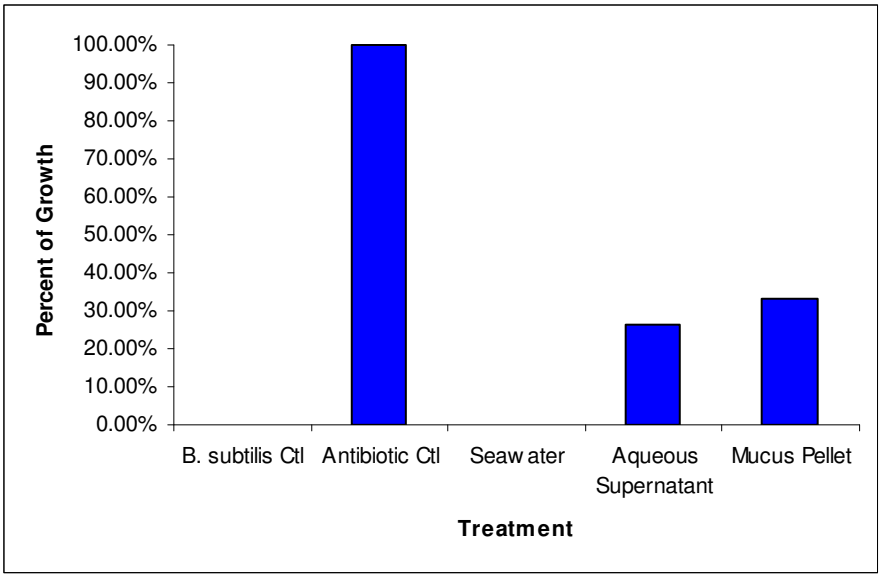

Figure 8. Percent reduction in growth of *B. subtilis* incubated with $Mg(TFA)_2$-extracted seawater, mucus aqueous supernatant, and mucus pellet. Assays were performed with *B. subtilis* cultures adjusted to an initial absorbance at 600 nm of 0.05. Penicillin/Streptomycin mix is used as an inhibitory control.

Task 3. Separation of bacterial symbionts from epidermal mucus

Subtask 3a. Separate bacterial symbionts from epidermal mucus

Continuing with the effort begun in Year 1, additional bacterial symbionts from mucus of both Atlantic stingrays and cownose rays were isolated in Year 2. Ninety-six bacterial isolates were cultured from *D. sabina* while 576 bacterial isolates were cultured from *R. bonasus* epidermal mucus.

Subtask 3b. Screen bacterial symbionts for antimicrobial activity using primary screen tester strains

In primary screens performed at Mote Marine Laboratory, 11 of the 96 *D. sabina* strains and 135 of the 576 *R. bonasus* strains isolated in Year 2 demonstrated antibiotic activity against at least one human pathogenic tester strain. All strains with antibiotic activity have been purified, culturable libraries have been cryopreserved, and cultures have been provided to project collaborators at the University of South Florida Center for Biological Defense. Sixty-six sequences have been prepared for 16S rDNA sequencing against the GenBank database for genetic identification. The 11 Atlantic stingray mucus-derived strains have been screened against additional sets of pathogenic bacterial strains at USF CBD, while the 135 cownose ray mucus-derived strains have been sent to USF and are in queue to be screened. Consequently, results of these screens are not available for inclusion in this Report.

Dasyatis sabina. Bacterial symbionts isolated from fresh *D. sabina* mucus yielded several strains which were assayed for antibiotic activity at Mote Marine Laboratory. Four of the strains showed broad spectrum activity against all of Mote's pathogen tester strains (Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, Vancomycin resistant *Enterococcus*, *Enterococcus faecalis*, and *Bacillus subtilis*), while the other seven strains had specific sensitivity against Vancomycin resistant *Enterococcus* (VRE).

In secondary screening at the University of South Florida Center for Biological Defense, susceptibility tests against a battery of CBD isolates (both BSL2 and BSL3 agents) including Bacillus spp (*B. anthracis* and *B. cereus*), *Enterococcus* sp*,* VRE*, Listeria, Staphylococcus* aureus (MSSA or MRSA), *Yersinia pestis, Acinetobacter* sp, *Pseudomonas aeruginosa*, *E. coli* (O157-H7 and nonO157), *Shigella* sp or *Salmonella* spp. were performed on the marine isolates. Six of the eleven strains were active against pathogenic strains not available at Mote (Table 2). A high priority in Year 3 will be to identify strains demonstrating activity in both primary screens at Mote and secondary screens at USF (such as strain 503-E7 in Table 2) and attempt to grow these isolates in quantities large enough to extract compounds for further examination of their anti-microbial properties. Pathogen overlay assay plates performed with Mote's pathogen tester strains showing zones of inhibition for the strains in Table 2 are shown in Figure 9. Magnified zones of inhibition for selected strains are shown in Figures 10 and 11.

Table 2. Primary and secondary screens of antibiotic activity of bacterial isolates from Atlantic stingray (*D. sabina*) mucus against pathogenic bacterial tester strains maintained at Mote Marine Laboratory (MML) and USF Center for Biological Defense (CBD).

Strain ID	Primary Screen (MML)	Secondary Screen (CBD)
530-E2	MSSA, MRSA, E. coli, VRE, *E. faecalis, B. subtilis*	
530-E6	MSSA, MRSA, E. coli, VRE, *E. faecalis, B. subtilis*	
530-E7	MSSA, MRSA, E. coli, VRE, *E. faecalis, B. subtilis*	*B. anthracis, Micrococcus* sp
530-E10	MSSA, MRSA, E. coli, VRE, *E. faecalis, B. subtilis*	*Micrococcus* sp.
530-A5	VRE	
530-B12	VRE	
530-C12	VRE	*B. anthracis*
530-D12	VRE	
530-E12	VRE	*Micrococcus* sp.
530-F11	VRE	*Micrococcus* sp.
530-F12	VRE	*Enterococcus. faecium*

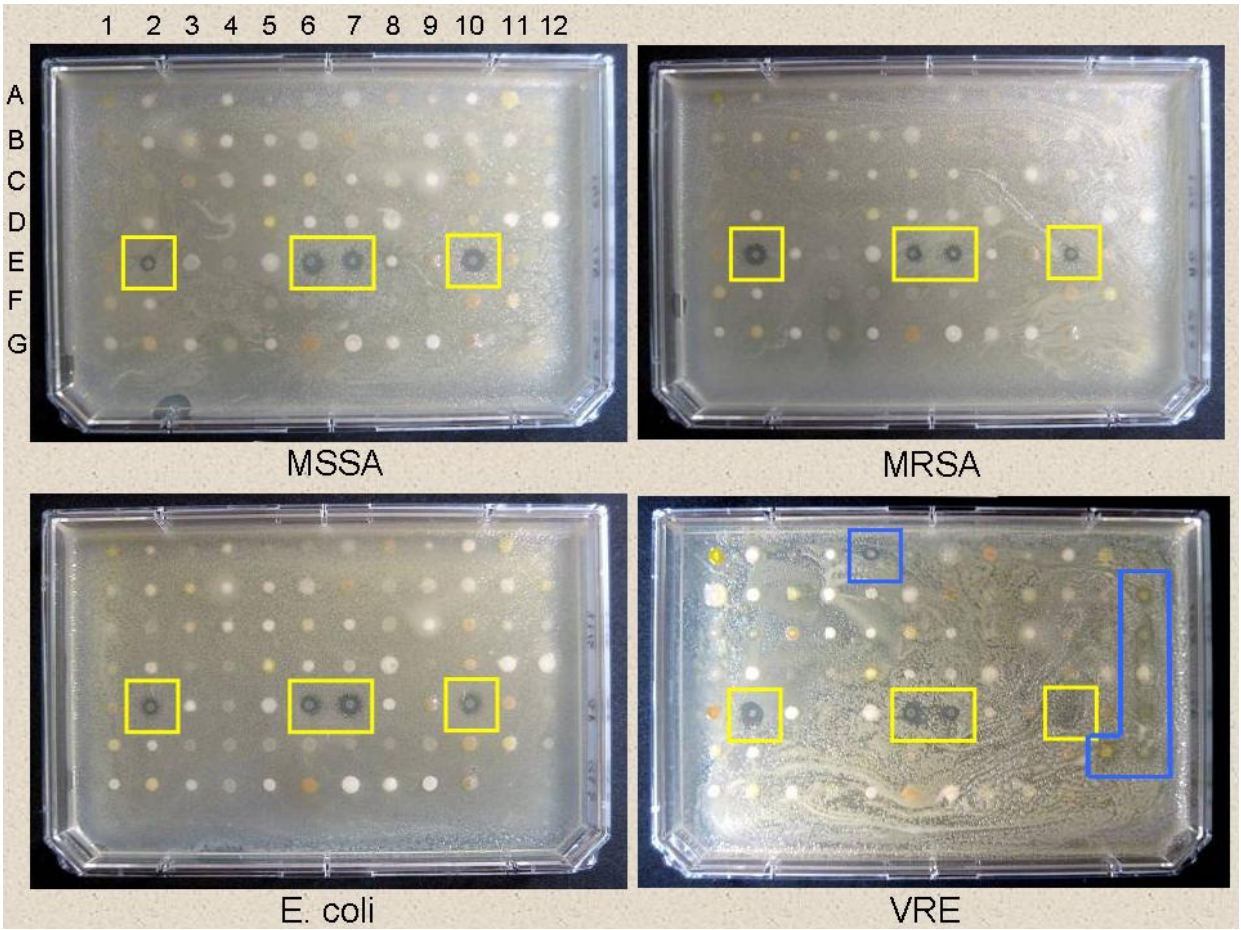

Figure 9. Pathogen overlay assay plates showing zones of inhibition for the strains in Table 2. The yellow-highlighted wells indicate the mucus-derived bacterial strains that displayed sensitivities against all Mote's pathogen tester strains, while the blue-highlighted wells indicate the strains that were specific for VRE.

Rhinoptera bonasus. Results of primary screening of antibiotic activities for the 135 cownose ray mucus-associated bacterial isolates are shown in Table 3. Of the 135 isolates with antibiotic activities, 29 have activities against MRSA, 3 against VRE and 13 against MSSA. Twenty-five isolates have activities against 2 or more tester strains, indicating the production of a potential broad spectrum antibiotic. Plate 803 is shown in Figure 10, with inhibitory zones for four strains shown in Figures 11 and 12.

Table 3. Antibiotic activity of bacterial isolates from cownose ray (*R. bonasus*) mucus against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory (MML).

Strain ID	Antibiotic Activity + Zone of inhibition (mm)
803 A6	B. subtilis (3) , MRSA (2.4)
803 A7	B. subtilis (3)
803 A8	B. subtilis (0.5)
803 A12	B. subtilis (0.75)
803 B6	MRSA (0.3) , B. subtilis (3.5) , MSSA (1.0)
803 B8	MRSA (0.1) , MSSA (0.2), B. subtilis (0.3)
803 B9	MRSA (0.2) , B. subtilis (1.2)
803 B11	B. subtilis (1.75)
803 C8	B. subtilis (0.1)
803 C9	B. subtilis (0.1)
803 C12	B. subtilis (0.1)
803 D5	MRSA (10.5)
803 D9	B. subtilis (1)
803 D10	MRSA (9.5) , MSSA (4.5)
803 E5	B. subtilis (8)
803 E6	MRSA 7.5 , VRE (4.5),MSSA (8.5), B. sutilis (10)
803 E8	B. subtilis(0.1)
803 F8	B. subtilis (0.1)
803 F10	B. subtilis (1)
803 G8	B. subtilis (0.5)
803 G9	B. subtilis (0.2)
803 G11	MRSA (6) , B. subtilis (7.5) , MSSA (4.5)
803 H9	B. subtilis (0.1)
803 H10	B. subtilis (0.1)
804 A10	MRSA (0.1)
804 C6	MRSA (1.25)
804 C10	MRSA (0.1)
804 D4	*B. subtilis*
804 D5	*B. subtilis*
804 D6	MRSA (1.25)
804 D11	B. subtilis (0.2), MRSA (0.1), MSSA (0.1)
804 E10	B. Subtilis 0.5 mm, MRSA 0.5 mm
804 E12	MRSA (2.5)
804 F3	MRSA (0.5)
804 F11	B subtilis (0.2), MRSA (0.1)
804 G3	MRSA (0.5)
804 G9	B. subtilis (8.0)

804 G10	B. subtilis (2.0), MRSA (1.0)
804 H2	B. subtilis (0.1)
804 H3	B. subtilis (0.75)
804 H4	B. subtilis (0.5)
804 H10	MRSA (0.1), B. subtilis (0.1)
805 A6	B. subtilis (0.2)
805 A9	B. subtilis (0.1)
805 A10	B. subtilis (0.75)
805 A12	B. subtilis (0.5)
805 B1	B. subtilis (0.1)
805 B9	B. subtilis (0.75)
805 B10	MSSA (0.1) , B. subtilis (0.1)
805 B12	B. subtilis (0.5)
805 C4	B. subtilis (0.1)
805 C5	B. subtilis (0.25)
805 C7	B. subtilis (1.5)
805 C8	B. subtilis (0.1)
805 C9	B. subtilis (0.75)
805 C10	B. subtilis (0.1)
805C12	B. subtilis (0.25)
805 D2	B. subtilis (0.1)
805 D6	B. subtilis (0.1)
805 D11	VRE (2.5) , B. subtilis (5.75)
805 D12	B. subtilis (0.1) , B. subtilis (0.1)
805 E7	B. subtilis (0.1)
805 E8	B. subtilis (4.5)
805 E11	B. subtilis (0.5)
805 E12	B. subtilis (0.1)
805 F2	B. subtilis (0.1)
805 F4	B. subtilis (0.1)
805 F8	B. subtilis (0.2)
805 F9	B. subtilis (0.2)
805 F10	B. subtilis (0.2)
805 F12	B. subtilis (0.1)
805 G2	B. subtilis (0.1)
805 G4	B. subtilis (0.1)
805 H2	B. subtilis (0.1) , B. subtilis (0.5)
805 H6	B. subtilis (0.1)
805 H7	B. subtilis (0.1)
805 H11	B. subtilis (0.1)
806 A10	B. subtilis (0.05)
806 A12	B. subtilis (0.1)
806 B9	B. subtilis (0.1)
806 B10	MSSA (>10)
806 B11	MSSA (>10)
806 B12	B. subtilis (0.2)
806 C4	B. subtilis (0.1)
806 C7	B. subtilis (0.5)
806 C9	B. subtilis (0.2)

806 C10	MSSA (>10), B. subtilis (0.2)
806 C11	MSSA (>10)
806 C12	B. subtilis (0.2)
806 D11	B. subtilis (6.5)
806 D12	B. subtilis (0.1)
806 E8	MSSA (1.5) , MRSA (2.0), B. subtilis (6.5)
806 E11	MSSA (0.1), MRSA (0.1) , B. subtilis (0.1)
806 F10	(MRSA 0.1 mm), (B. subtilis 0.2)
806 F12	B. subtilis (1.0)
807 A1	B. subtilis (0.1)
807 A2	B. subtilis (0.1)
807 A5	B. subtilis (0.1)
807 A6	B. subtilis (0.1)
807 A8	B. subtilis (0.1)
807 B5	B. subtilis (0.1)
807 B7	B. subtilis (0.1)
807 C6	B. subtilis (0.1)
807 D2	B. subtilis (0.1)
807 D8	B. subtilis (0.1)
807 E3	B. subtilis (0.2)
807 E5	B. subtilis (0.1)
807 E6	B. subtilis (0.1)
807 F1	B. subtilis (0.1)
807 F5	B. subtilis (0.1)
807 G1	B. subtilis (0.1)
807 G6	B. subtilis (0.1)
807 H1	B. subtilis (0.1)
807 H7	VRE (1.5)
807 H8	VRE (0.5)
808 A7	B. subtilis (0.5)
808 A12	MRSA (0.05), B. subtilis (0.2)
808 B1	B. subtilis (0.25)
808 B12	B. subtilis (0.1)
808 C1	B. subtilis (0.25)
808 C10	B. subtilis (0.1)
808 D1	MRSA (0.1), B. subtilis (0.5)
808 D5	B. subtilis (1.0)
808 D12	B. subtilis (0.05)
808 E1	B. subtilis (0.1)
808 E2	MRSA (0.1), B. subtilis (0.5)
808 E8	B. subtilis (0.1)
808 E9	B. subtilis (0.5)
808 E12	B. subtilis (0.05)
808 F11	MRSA (0.2), B. subtilis (0.1)
808 G1	MRSA (0.1), B. subtilis (0.2)
808 G11	B. subtilis (1.5)
808 G12	B. subtilis (0.2)
808 H4	B. subtilis (0.1)
808 H5	B. subtilis (0.1)

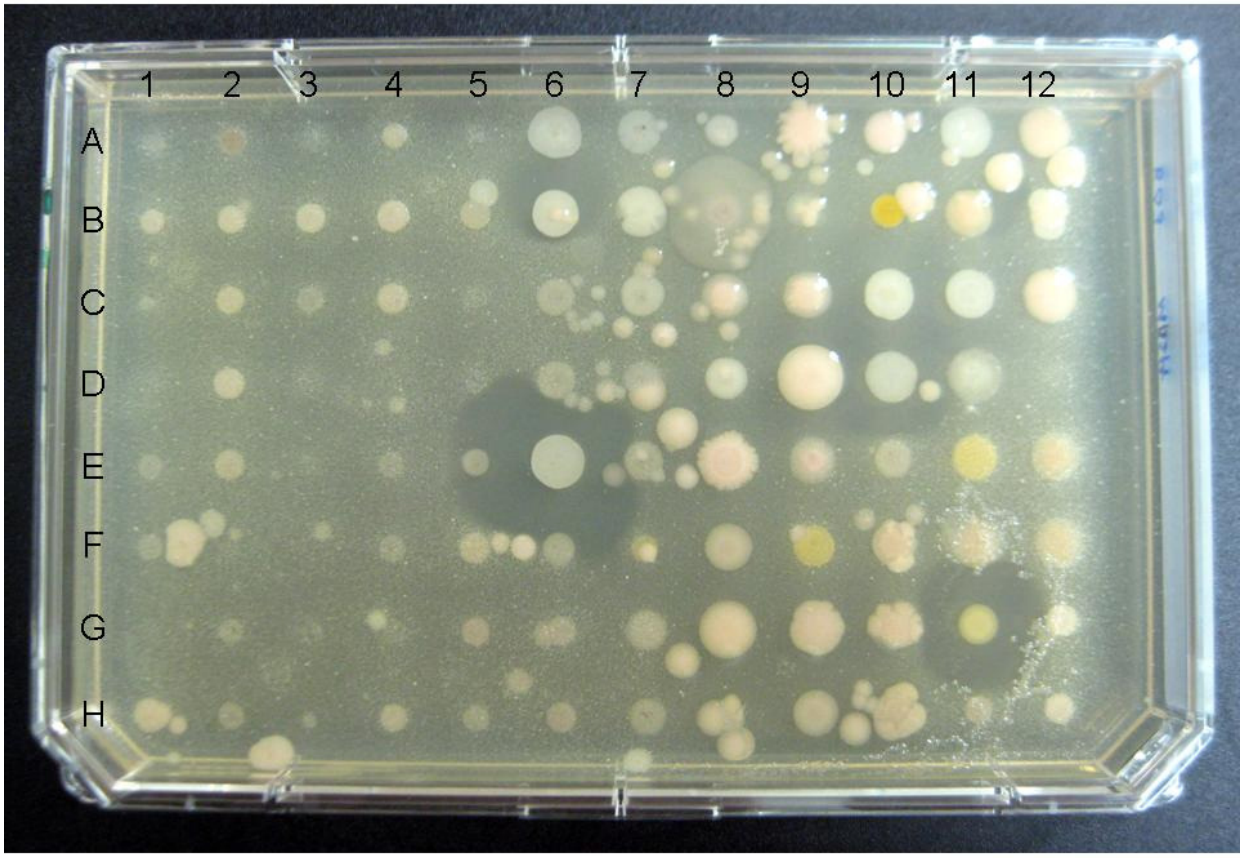

Figure 10. Photograph of Plate 803 containing 96 of the 578 bacterial strains cultured from *R. bonasus* mucus overlaid with methicillin resistant *S. aureus* (MRSA). Zones of inhibition are visible in 7 of the 96 isolates assayed on this plate.

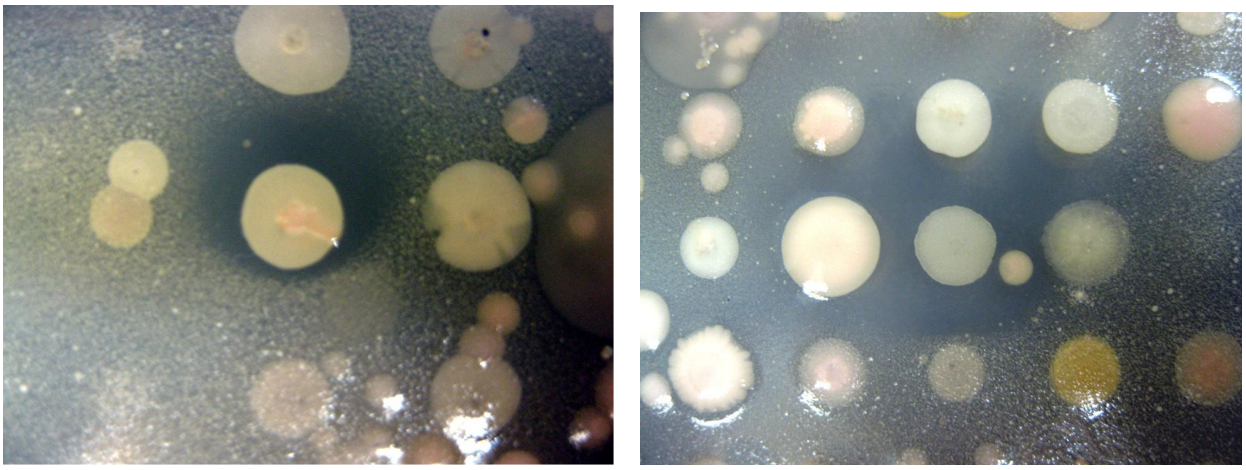

Figure 11. Higher magnification photographs of zones of inhibition for bacterial strains in positions B6 (left) and D10 (right) in Plate 803 (Figure 10). Isolates were overlaid with methicillin resistant *S. aureus* (MRSA).

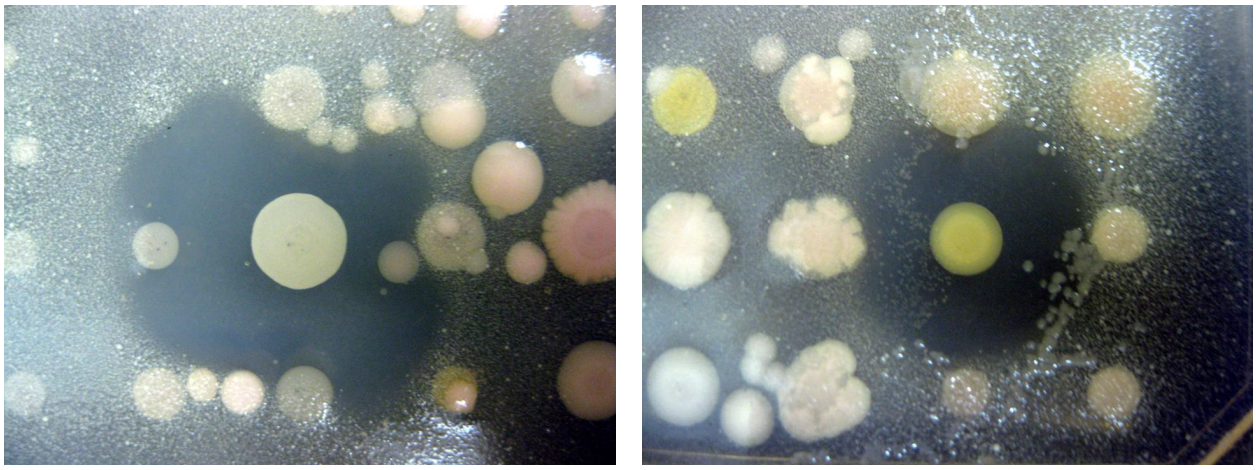

Figure 12. Higher magnification photographs of zones of inhibition for bacterial strains in positions E6 (left) and G11 (right) in Plate 803 (Figure 10). Isolates were overlaid with methicillin resistant *S. aureus* (MRSA).

Task 4. Determine contribution of epidermal mucus to wound healing in elasmobranchs (Months 6 - 30).

Subtask 4a. Experimental wounding of animals
During Year 2, experimental wounding studies related to Aim 2 (subtasks 4a and 4b) and Aim 3 (subtasks 5a, 5b, and 5c) were initiated. The Atlantic stingray, *Dasyatis sabina*, is the ray of choice for these studies. Because of their size (smaller than cownose rays) and their sedentary and solitary behavior (not found in large schools like cownose rays), Atlantic stingrays are ideal for long-term captive maintenance in smaller tanks compared to those required for cownose rays, and can be easily manipulated for experimental procedures.

Based on pilot studies and input from collaborators at Daemen College, it was determined that circular wounds would be the best to analyze. Using autoclaved circular brass coring tools, circular wounds penetrating the epidermal and dermal layers of the skin can be inflicted, exposing the underlying epaxial musculature.

In wounding experiments performed during Year 2, wounds ranged from 1.0 to 2.0 cm in diameter. At regular intervals, digital photographs were taken with the progression of wound healing documented in comparison to a reference "target". While collaborators at Daemen College confirmed that circular wounds were preferred over square or rectangular wounds, a diameter of 2 cm was determined to be too large for healing to progress at a useful rate for analysis. As a result, circular wounds reduced in size to a range of 1.0 to 1.5 cm diameter have been surgically inflicted on *D. sabina* specimens. At weekly intervals, mucus in the area of the wounds was collected and digital photographs were taken and sent to Daemen College for analysis using wound measurement software to calculate healing trajectories. The weekly progression of wounds with reference targets for two rays for 56 days is shown in Figure 14.

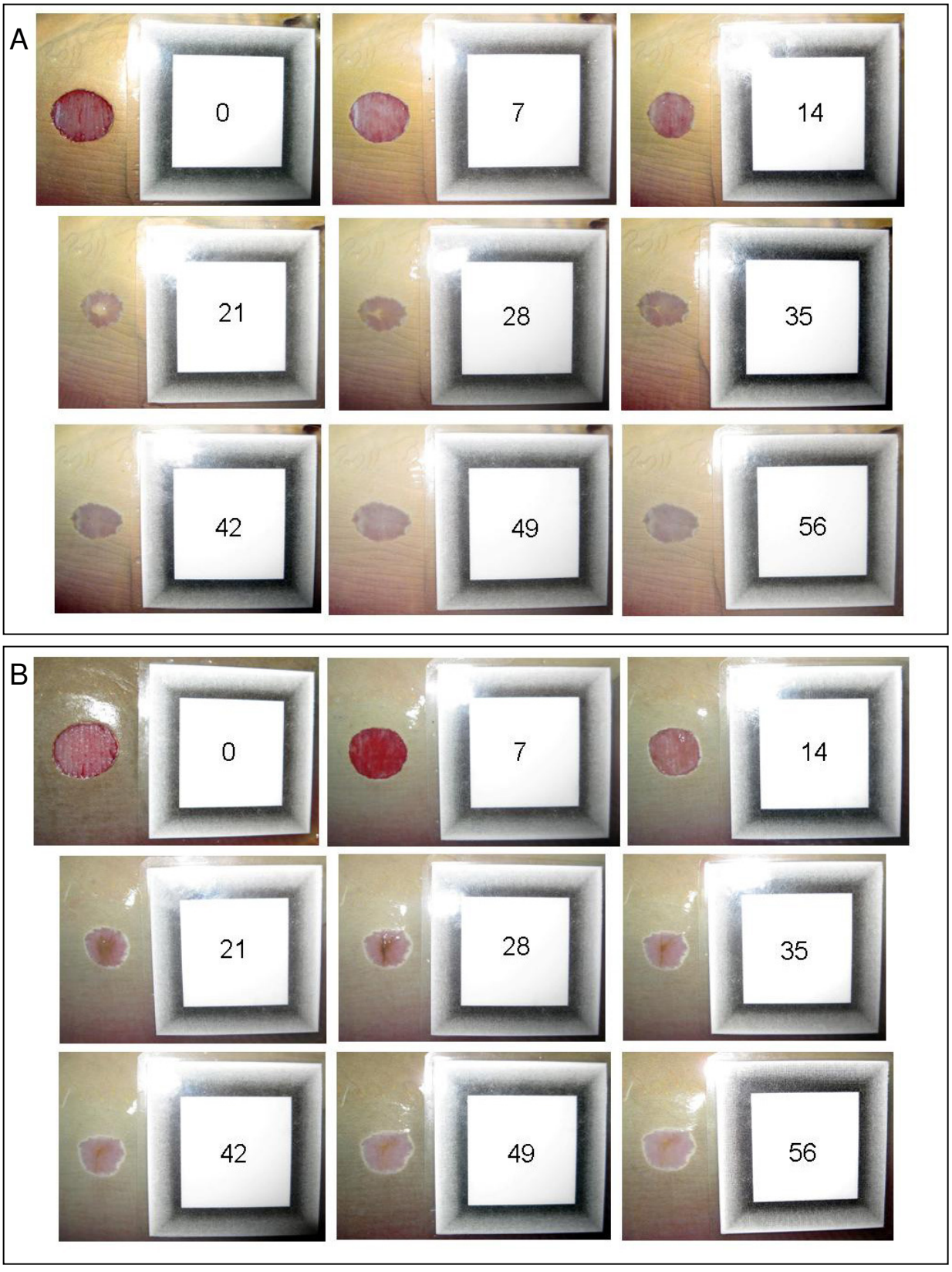

Figure 14. 1.5-cm diameter circular wounds and reference targets, showing the progression of wound healing in two Atlantic stingrays, *Dasyatis sabina* (A & B). Numbers superimposed on each target represent time in days.

Experimental wounding studies result in infection-free healing in the form of scar tissue that forms across the wound. With weekly documentation, an observation that has been remarkably consistent is the appearance of fibrous tissue in the center of the wounds at the three week timepoint, which gradually dissipates to a uniform scar tissue across the wound. This can be seen more easily in Figure 15, where wounds are photographed with a centimeter scale, allowing a magnified view of the healing process. An interesting observation is that the pigment in the fibrous tissue reflects the pigment in the skin for a particular ray (yellowish in lighter pigmented rays and brownish in darker rays).

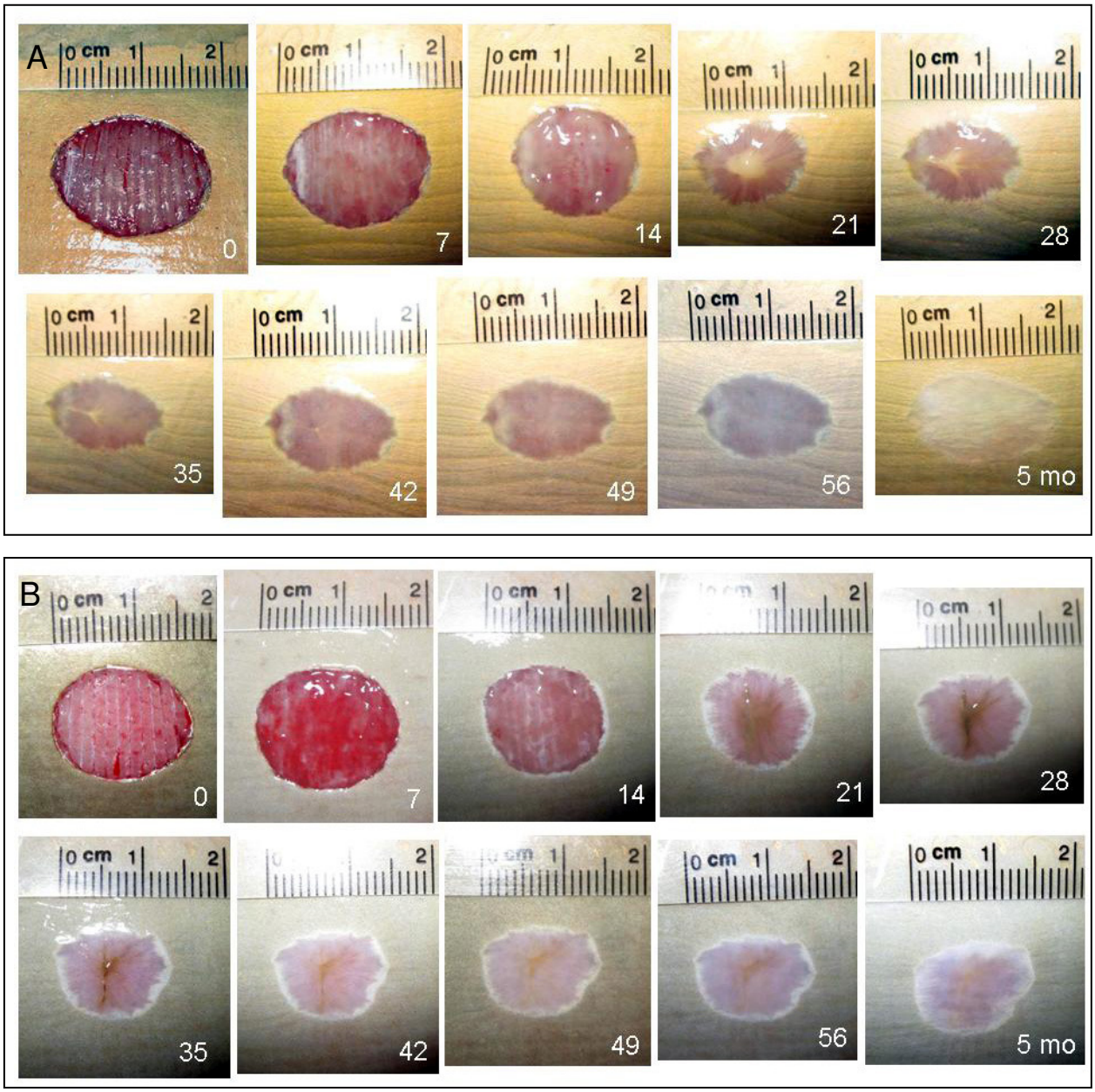

Figure 15. Higher magnification of circular wounds in Figure 14. Sizes of images have been adjusted so that the scale is the same for each set of wounds (A & B). Numbers superimposed on each image represent time in days.

Experimental wounding studies with 1.0 cm circular wounds (Figure 16) utilized narrower intervals of documentation to focus on the appearance of tissue on the wound surface. As previously described, experimental wounds penetrating the epidermal and dermal layers of the skin were inflicted, exposing the underlying epaxial musculature. Wound surfaces were also sampled with a sterile swab, immediately frozen, and stored at -80 ℃ for analysis at Daemen College of potential biomarker molecules associated with the healing process. Digital images documenting the progress of wound healing were taken at the time of surgery (day 0) and at days 7, 10, 13, 15, 17, 20, 22, 24 and 27 post-surgery.

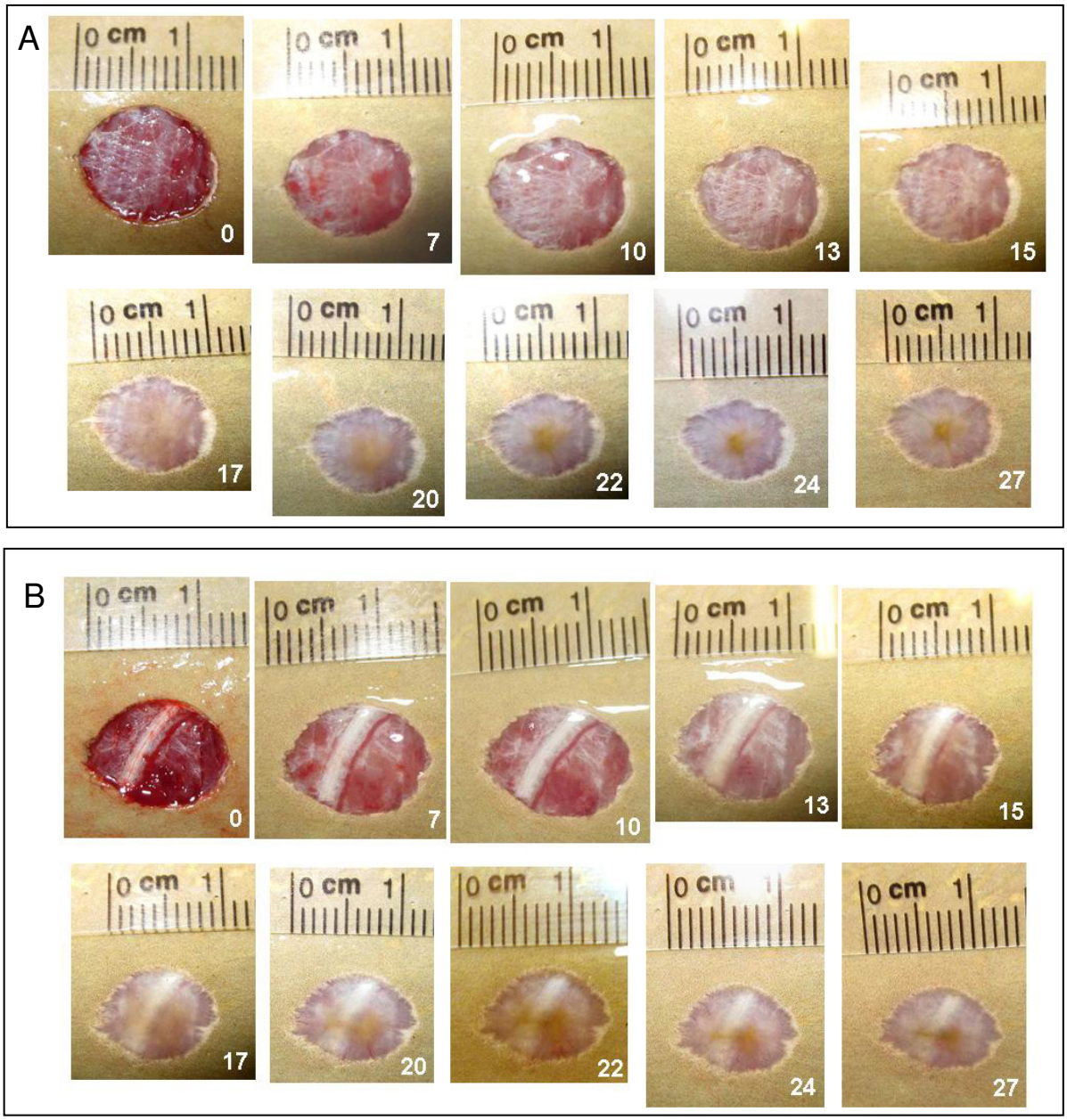

Figure 16. 1.0-cm diameter circular wounds, showing the progression of wound healing in two Atlantic stingrays, *Dasyatis sabina* (A & B). Numbers superimposed on each image represent time in days.

At the narrower intervals used in this study, the appearance around three weeks of centrally located fibrous tissue begins to be visible by day 17, with greatest accumulation at day 20, followed by the anticipated dissipation across both wounds.

Initial examination of wound surface swabs collected during this study, however, indicated that this technique may not be collecting sufficient material to analyze wound healing biomarkers. No measurable protein was isolated from 17 of 18 swabs processed with negligible protein (0.038 mg/ml) isolated from the final swab. While this technique is standard protocol for sampling wound exudates in humans, the viscous nature of stingray mucus may prevent the swab tip material from absorbing enough wound surface material. For future wound healing studies focusing on proteomic analysis of wound-associated mucus, multiple wound sites per animal will be designed. This will increase the surface, both on and around wounds, from which mucus can be collected.

At 5 weeks of healing, the experimental wounds in Figure 16 were biopsied for histology. As with all previous studies, healing was absolutely free of any inflammation or apparent infection. The biopsies bisected the healed wounds and included areas of normal skin, as well as portions of the underlying musculature (Figure 17). These biopsy procedures are not life-threatening, with experimental animals exhibiting normal behavior and feeding activity following revival from anesthesia.

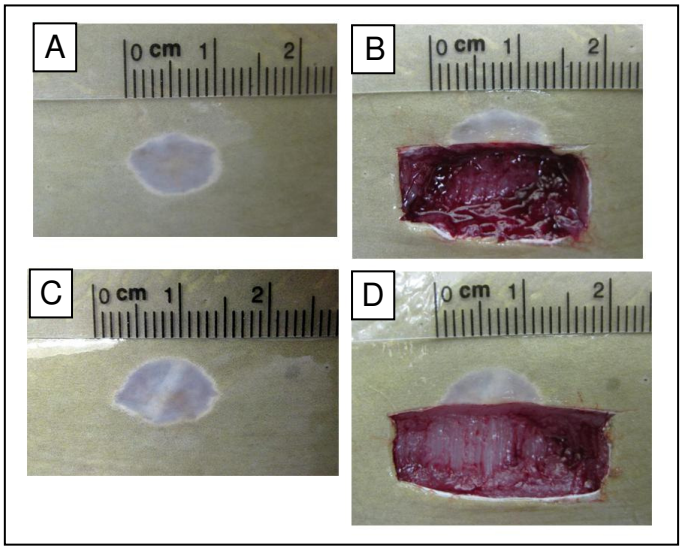

Figure 17. Five-week wounds (A & C) were biopsied for preliminary histological analysis. Biopsies (B & D) bisected the healed wounds and included areas of normal skin, as well as portions of the underlying musculature.

Preliminary histological examination of the biopsied wounds and surrounding tissue indicated that the epidermis remained distinct from the unwounded skin with respect to its pigmentation and allowed the wound margin to be identified in the fixed tissue and prepared histological section. The structure of ray skin includes three main layers that lead to underlying musculature (Figures 18 and 19). The epidermis is outermost and contains secretory mucus cells. The dermis is divided into two layers, a loose stratum spongiosum and a more compact stratum compactum. The hypodermis separates the dermis from the muscle and is composed of loose connective tissue.

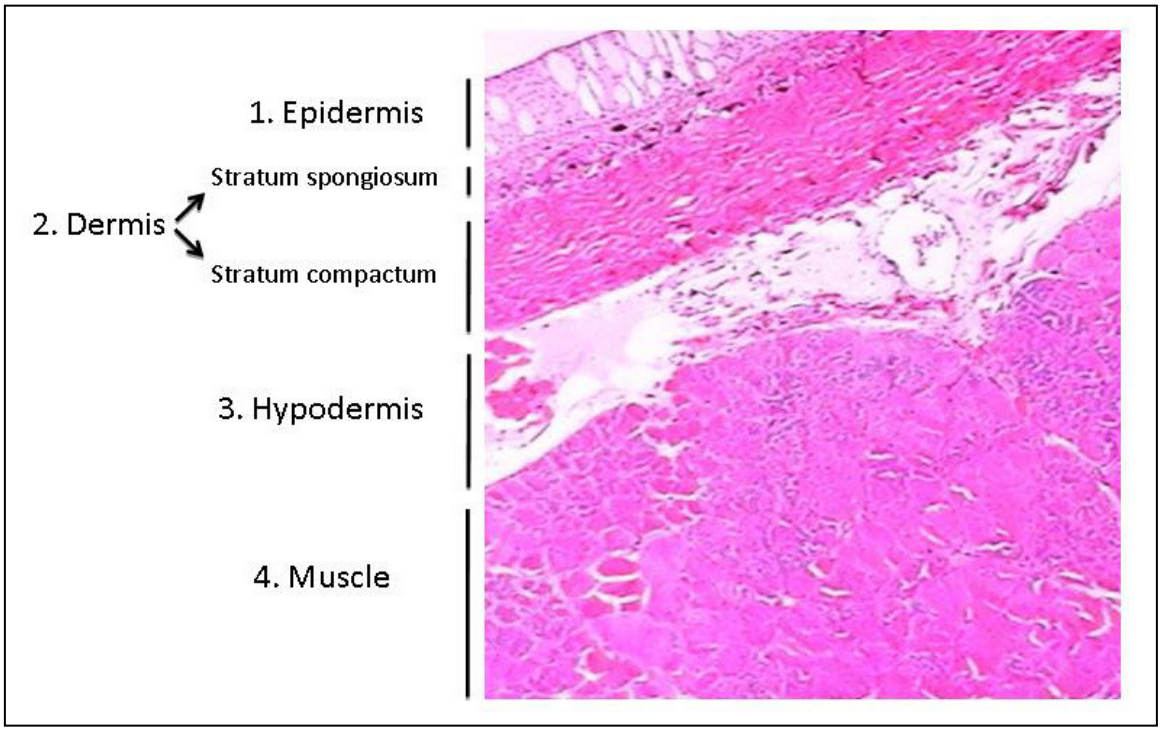

Figure 18. The structure of normal Atlantic stingray skin, showing epidermal, dermal and hypodermal layers typical of vertebrate skin histology.

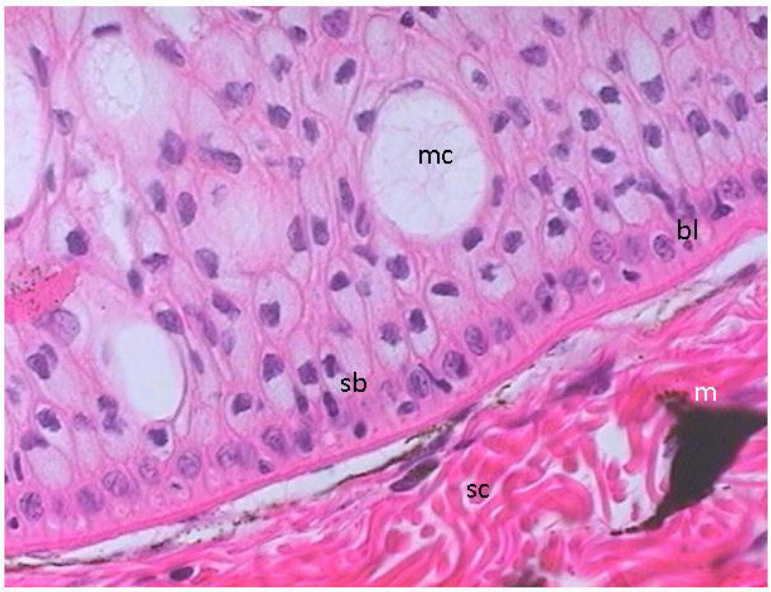

Figure 19. Normal Atlantic stingray epidermis contains mucus cells (mc) and a well defined basal lamina (bl). The stratum basale (sb) cells are columnar and organized in a single layer. The stratum spongiosum underlies the basal lamina and is nominal in comparison to the stratum compactum (sc). Melanophores (m) can be observed within the dermis.

Histology of the five-week wound and surrounding normal tissue is shown in Figure 20. The wound shows epithelial hyperplasia with concomitant loss of underlying dermal architecture. The epidermis has mucus cells but their distribution is not regular across the scar tissue. The area under the epidermis is interrupted with localized accumulations of blood (possibly as a result of bisecting the tissue during biopsy) and interstitial fluid bordered by musculature. Although the wound is closed it is not fully restructured. Mucus cells are abundant in the hypertrophied epidermis. At the wound margin there is a multi-layered stratum basale with cells nearly cuboidal in form rather than columnar. The stratum basale and stratum compactum are oriented perpendicular to the original wound edge. The basal lamina is not identifiable and cells with basophilic extensions populate the margin between the epidermis and dermis. There is no discernible stratum spongiosum.

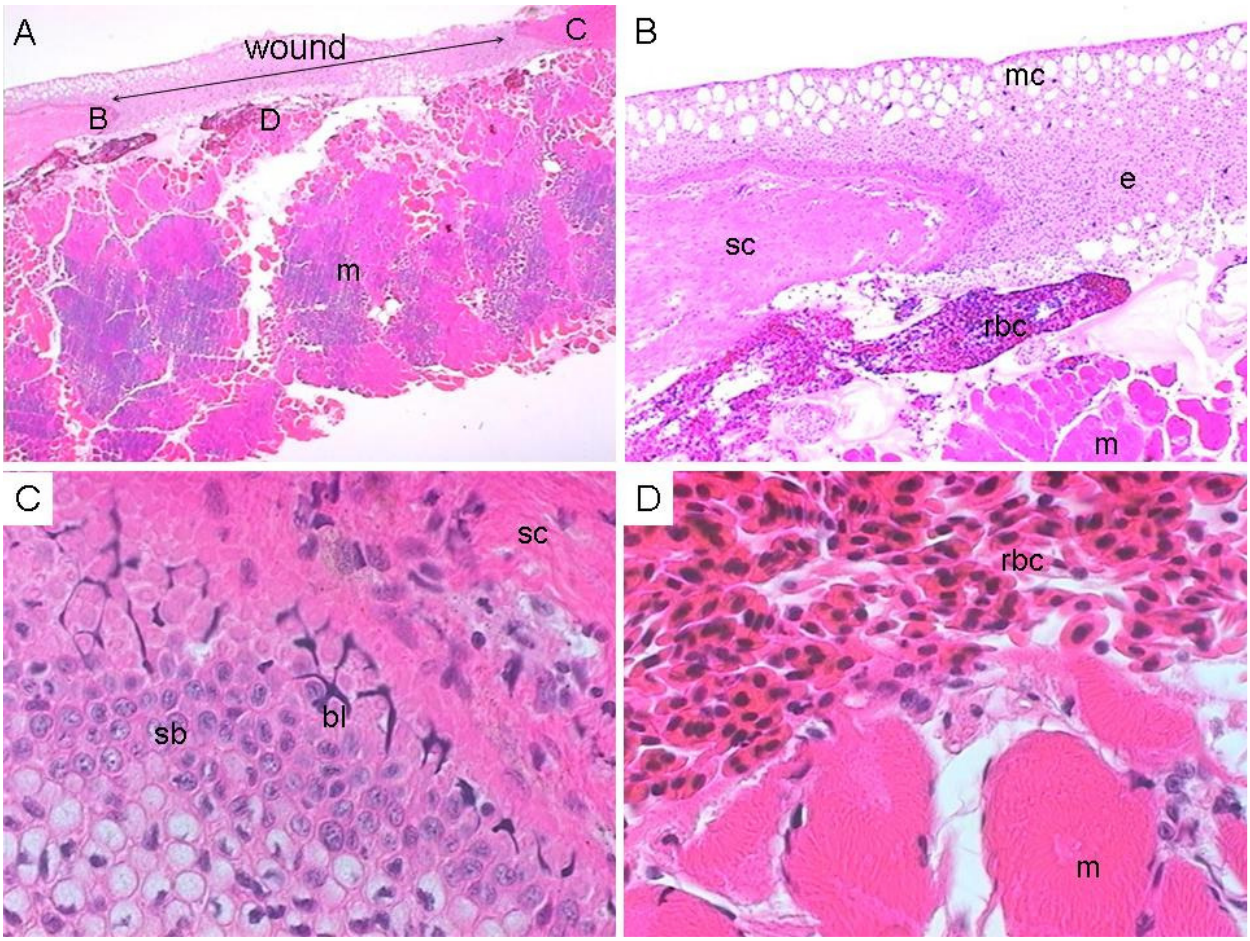

Figure 20. Histology of the biopsied tissue including the wound after 5 weeks and surrounding normal tissue. The wound area is easily recognized by the interrupted epidermal structure present across nearly the entire view (Figure 20 A). The letters B, C and D indicate positions of the enlarged views in Figures 20 B, C, and D.
m, muscle; **e**, epithelial hyperplasia; **rbc**, red blood cells; **mc**, mucus cells; **sb**, stratum basale; **sc**, stratum compactum; **bl**, basal lamina.

The experimental wounding studies to date have focused on characterizing the progression of healing events and healing timeline. With the biopsy histology in Figure 20 representing an essentially healed wound, an experimental wounding study to focus on histology and healing biomarkers associated with the formation and dissipation of the centralized fibrous tissue was performed. Four experimental wounds 1.0 cm in diameter were inflicted, with tissue biopsies obtained at 17, 21, 24, and 28 days of healing. Biopsies were bisected, with one half fixed in 10% formalin and one half frozen at -80° C. Tissues were sent to Daemen College for analysis. These analyses are in progress and results are not available for inclusion in this report.

Task 6. Isolate bioactive compounds in epidermal mucus.

During Year 2, efforts to isolate mucus compounds explored the potential to separate compounds based on their isoelectric points and by their extent of glycosylation.

a) Isoelectric Focusing (IEF):
In the technique called isoelectric focusing, molecules are separated by differences in their isoelectric points (pI). Mixtures of proteins are subjected to a gel in which a voltage-induced pH gradient is established. Individual proteins in the mixture migrate through the gradient until they reach a pH at which they are isoelectric (i.e., no net charge in the solvent being used). Isoelectric focusing of Atlantic stingray mucus-derived proteins is shown in Figure 21.

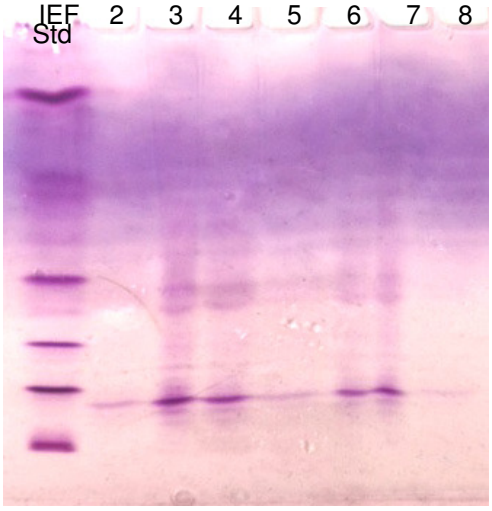

Figure 21. IEF was conducted on Criterion IEF gels (Bio-Rad) with a pH range of 3-10. Gels contained 2% ampholytes and were run in a Bio-Rad electrophoresis/IEF unit using a lysine-arginine cathode solution and a phosphoric acid anode solution. Run time ~ 2 ½ hrs at gradually increasing voltage from 100 to 500 volts. Gels were stained with 0.04% coomassie brilliant blue/0.05% crocein scarlet in 27% isopropanol / 10% acetic acid. The stained proteins carry the more deep magenta /red of the crocein scarlet. Lane 1: IEF Standard proteins; Lanes 2-8: varying concentrations of mucus protein mixture.

b) Chromatofocusing:
Efforts to separate mucus components using chromatofocusing were initiated by collaborators at Clemson University. Chromatofocusing is a protein-separation technique that allows resolution of proteins in a complex mixture according to differences in their isoelectric point. Chromatofocusing pH ranges were determined based on IEF values and narrower gradients were performed as protein peaks were detected by scans at 280 nm.

Samples were applied to a column of PBE 94 (6 cm x 0.9 cm) polybuffer anion exchanger equilibrated against starting buffer (0.025 M piperazine at the appropriate starting pH), followed by titration of the anion exchanger with polybuffer 74 HCl at the appropriate end pH. One mL fractions were collected until the pH of the eluent was the designated pH in two consecutive fractions. A chromatofocusing column profile of mucus compounds is shown in Figure 22.

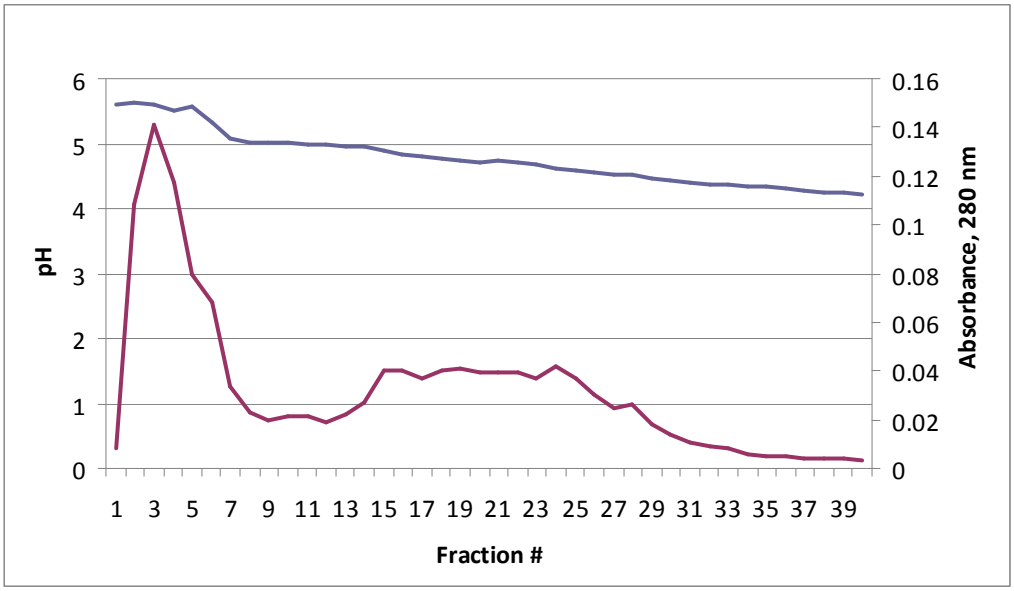

Figure 22. Column profile of mucus proteins separated on a PBE 94 polybuffer anion exchanger. The pH gradient ranged from pH 5.6 to 4.0. The column was monitored at 280 nm and 1.0 mL fractions were collected and fractions were pooled for further analysis.

c) Analysis of Carbohydrate-containing Mucus Proteins:
Previous studies characterizing the molecular components of stingray mucus have utilized techniques that stain specifically for protein. With the possibility that some of these proteins are glycosylated (carbohydrate-containing), mucus proteins were separated under both native (Figure 23) and denaturing (Figure 24) conditions and stained with a glycoprotein staining kit (G Biosciences). Gels analyzed using this kit are first stained for glycosylated proteins/subunits which appear as magenta colored bands. The same gel is then stained with coomassie blue to stain for protein.

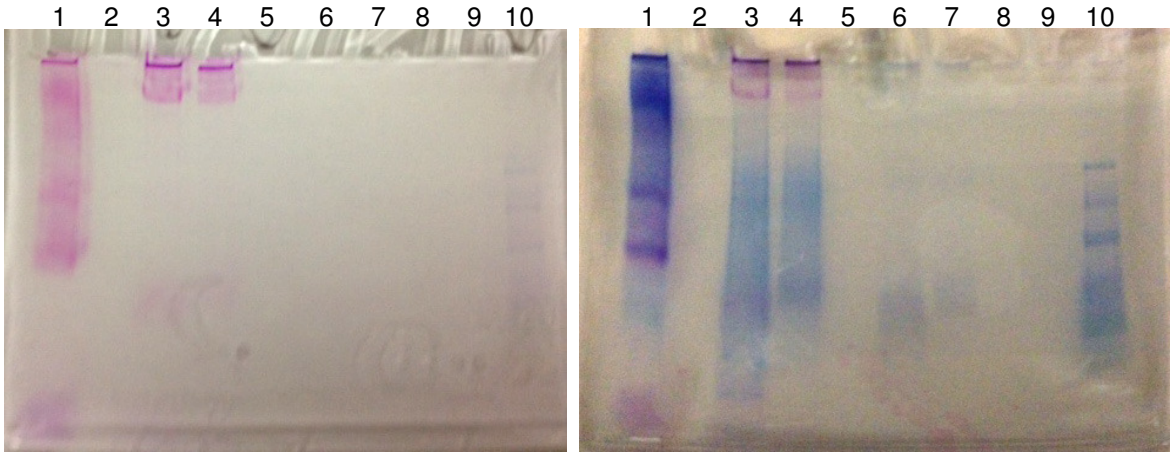

Figure 23. Native gel (4% acrylamide) stained with glycoprotein staining kit from G Biosciences. Left: Carbohydrate stain; Right: Protein stain. Lane 1: Glycoprotein positive control; Lanes 3 and 4: Mucus Aqueous Supernatant; Lanes 6 and 7: Pooled fractions from chromatofocusing; Lane 10: Protein standards. Lanes 2, 5, and 9 are empty.

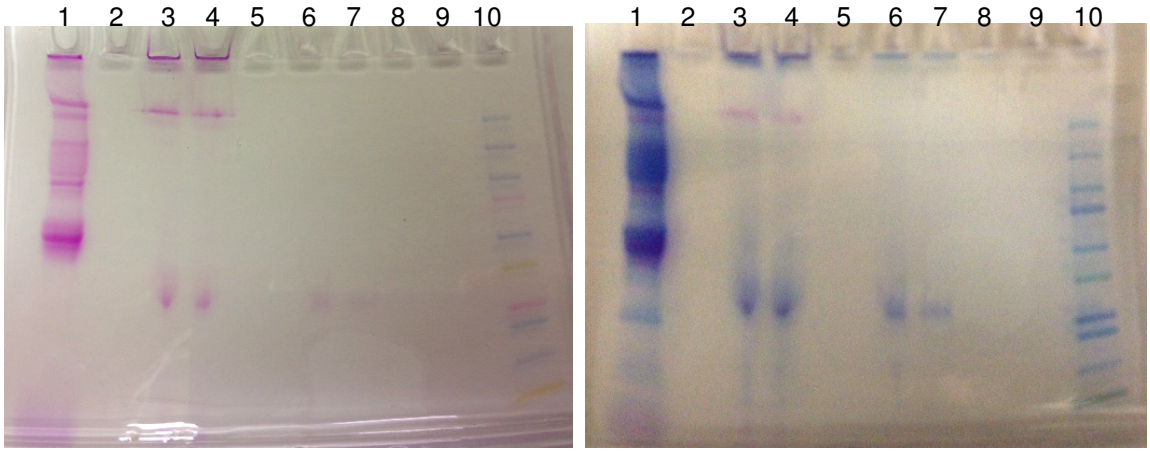

Figure 24. SDS polyacrylamide gel (4-20% gradient gel) stained with glycoprotein staining kit from G Biosciences. Left: Carbohydrate stain; Right: Protein stain. Lane 1: Glycoprotein positive control; Lanes 3 and 4: Mucus Aqueous Supernatant; Lanes 6 and 7: Pooled fractions from chromatofocusing; Lane 10: Protein standards. Lanes 2, 5, and 9 are empty.

Since several of the mucus proteins were shown to be glycosylated, studies during Year 3 will include fractionation and purification of carbohydrate-containing proteins by lectin affinity chromatography.

KEY RESEARCH ACCOMPLISHMENTS:

- Thermal stability studies indicate that the structural integrity of mucus-associated proteins is stable following prolonged storage and freeze-thaw cycles.

- Methods utilized to extract mucus compounds focusing on non-denaturing procedures have been successful in obtaining mucus pellet extracts enriched in low molecular weight compounds. Of particular interest is magnesium trifluoroacetate, a neutral salt that extracts mucus pellet compounds with low but measurable antibiotic activity.

- 96 additional bacterial isolates have been purified from *D. sabina* epidermal mucus and culturable libraries have been cryopreserved. In primary screens performed at Mote Marine Laboratory, 11 of the 96 strains demonstrated antibiotic activity against at least one human pathogenic tester strain. Four of the strains showed broad spectrum activity against all of Mote's pathogen tester strains, while the other seven strains had specific activity against VRE. Of these 11 strains, six demonstrated antibiotic activity against at least one wound infection pathogen strain in screens performed at University of South Florida Center for Biological Defense.

- 576 additional bacterial isolates have been purified from *R. bonasus* epidermal mucus and culturable libraries have been cryopreserved. In primary screens performed at Mote Marine Laboratory, 135 of the 576 strains showed antibiotic activity against at least one human pathogenic tester strain.

- Experimental wounding studies initiated during Year 2 developed procedures to inflict, observe, photograph, and biopsy wounds. Without exception healing was absolutely free of any inflammation or apparent infection.

REPORTABLE OUTCOMES:

Presentations

- Gordon Conference: Oceans and Human Health. Carl Luer, Catherine J. Walsh (presenter), Kim Ritchie, Jodi Miedema, Jennifer Yordy, A.B. Bodine, Andrew Cannons, Vicki Luna, Laura Edsberg, Jen Wyffels. "Epidermal mucus from cownose ray (*Rhinoptera bonasus*) and Atlantic stingray (*Dasyatis sabina*): Antimicrobial properties and role in wound healing" University of New England. Biddeford, ME. June 3-8, 2012.

- Invited lecture. Carl Luer (presenter), "Antimicrobial Properties of Epidermal Mucus from Two Species of Ray (Cownose Ray, *Rhinoptera bonasus*, and Atlantic Stingray, *Dasyatis sabina*)". Ecology of Sharks Class at University of North Florida, Jacksonville, FL. June 29, 2012.

- Annual Meeting of the American Elasmobranch Society. Carl Luer (presenter), Cathy Walsh, Kim Ritchie, Jennifer Yordy, Jodi Miedema, A.B. Bodine, Andrew Cannons, Vicki Luna, Laura Edsberg and Jen Wyffels. "Antimicrobial Properties of Epidermal Mucus from Two Species of Ray (Cownose Ray, *Rhinoptera bonasus*, and Atlantic Stingray, *Dasyatis sabina*)." University of British Columbia, Vancouver, British Columbia, Canada, August 8-14, 2012.

Request for Research Data

- Selected to be part of a portfolio briefing to Assistant Secretary of Defense for Health Affairs ASD(HA). To be highlighted or as back-up information to demonstrate the depth of the CDMRP's portfolio. Powerpoint slides provided to CDMRP Science Officer, Dwayne L. Taliaferro, Ph.D., on October 10, 2012.

Databases

- The worldwide database GenBank is used for BLAST searches performed on 16S rDNA sequence data generated from stingray epidermal mucus.

Conclusions

Epidermal mucus collected passively from the surface of stingrays contains proteins in both an aqueous supernatant portion and in a viscous pellet that separates from the aqueous portion upon sitting or via centrifugation. As visualized on SDS polyacrylamide gels, mucus proteins range in molecular weight from approximately 6,000 to 200,000 daltons. Temperature stability studies indicate that the structural integrity of mucus-associated proteins is stable following prolonged storage and freeze-thaw cycles.

Since methods to assess antimicrobial activity of chemically extracted material have been hampered by difficulty in removing residual chemicals (i.e., acids and organic solvents) involved in extraction procedures, methods during Year 2 focused on non-denaturing compounds, the most promising being the magnesium salt of trifluoroacetic acid. Extractions using this neutral salt were successful in obtaining extracts of mucus pellets enriched in low molecular weight compounds demonstrating low but measurable antibiotic activity.

Epidermal mucus also contains a natural flora of symbiotic bacteria, which tend to be concentrated in the mucus pellet. Culturable bacteria from the mucus of two species of stingray (cownose rays, *Rhinoptera bonasus*, and Atlantic stingrays, *Dasyatis sabina*) have been isolated and screened for antimicrobial activity at Mote Marine Laboratory (MML) using a primary screening panel of pathogenic bacterial tester strains, including Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA), *Escherichia coli*, Vancomycin resistant *Enterococcus* (VRE), *Enterococcus faecalis*, and *Bacillus subtilis.* Of 576 bacterial isolates cultured from cownose ray epidermal mucus during Year 2, 135 strains demonstrated antibiotic activity against at least one human pathogenic tester strain. Of 96 bacterial isolates cultured from Atlantic stingray mucus, 11 strains demonstrated antibiotic activity against at least one human pathogenic tester strain.

Mucus-associated bacterial isolates have also been provided to project co-investigators at University of South Florida Center for Biological Defense (CBD), where cultures have been screened against a different panel of human pathogenic tester strains, including Bacillus spp (*B. anthracis* and *B. cereus*), *Enterococcus* sp*,* VRE*, Listeria, Staphylococcus* aureus (MSSA or MRSA), *Yersinia pestis, Acinetobacter* sp, *Pseudomonas aeruginosa*, *E. coli* (O157-H7 and nonO157), *Shigella* sp or *Salmonella* spp. Of the 11 Atlantic stingray mucus-associated bacterial isolates demonstrating antibiotic activity from the MML screening procedures, 6 strains demonstrated antibiotic activity against at least one human pathogenic tester strains in the CBD panel. A high priority in Year 3 will be to identify strains demonstrating activity in both primary screens at Mote and secondary screens at USF and attempt to grow these isolates in quantities large enough to extract compounds for further examination of their anti-microbial properties.

Experimental wounding studies initiated during Year 2 resulted in wounds that healed without infection or inflammation. A consistent observation is the appearance of raised

fibrous tissue in the center of the wounds by the three week timepoint, which gradually dissipates to a uniform scar tissue across the wound. Preliminary histology on essentially “healed” wounds provided valuable baseline data on restructuring of epidermal and dermal tissue during the healing process.

Evaluation of the knowledge:

Since this funded program focuses on basic research, development of a medical “product” is not within the scope of this project. However, the antimicrobial activity demonstrated by numerous mucus-associated bacterial isolates holds great promise for the identification of antibiotic compounds and future development of therapeutics to treat wounds sustained on the battlefield.

APPENDICES:

Appendix 1. Preparation of 1 M magnesium trifluoroacetate

Appendix 2. Spectrophotometric antimicrobial assay: *Bacillus subtilis*

Appendix 3. Procedure for antibiotic screening of cultured libraries (Mote Marine Laboratory)

Appendix 4. Procedure for antibiotic screening of cultured libraries (University of South Florida Center for Biological Defense)

Appendix 5. Meeting abstracts

1) Gordon Conference: Oceans and Human Health. University of New England. Biddeford, Maine. June 3-8, 2012.

2) Annual Meeting of the American Elasmobranch Society. University of British Columbia, Vancouver, British Columbia, Canada, August 8-14, 2012.

Appendix 1. Preparation of 1 M magnesium trifluoroacetate

In a fume hood, place 50 mL of water in a 250mL beaker and support beaker in ice. After about 10 minutes, slowly and carefully add 15.2 mL (22.8 grams) of trifluoroacetic acid. The solution will fume vigorously. After 5 minutes, add a total of 4 grams of magnesium oxide in 4 or 5 equal portions with swirling or magnetic stirring. The reaction is mild if the temperature remains below 5° C. After the addition of the MgO, allow the reaction mixture to warm to room temperature with stirring. When the temperature is approximately room temperature, dilute to near 100 mL, check the pH, and adjust with small portions of MgO or trifluoroacetic acid until the pH is between pH 7 and pH 8. (Note: it usually takes ~0.1 gram or so of MgO to bring the pH to slightly less than 8.) Filter the solution. This will produce 0.1 mole of $Mg(TFA)_2$ in 100 mL of water = 1 M.

Appendix 2. Spectrophotometric antimicrobial assay: *Bacillus subtilis*

1) Grow *Bacillus subtilis* overnight at room temperature, shaking (one to three colonies of actively growing plate cultures into 5 mL Luria Broth).

2) To prepare a working *B. subtilis* stock, pellet 3 mL of mid-log phase cultures (A_{600} of 0.4 ± 0.1) by centrifugation at 500*g* for 10 minutes at 4ºC. Wash 2 times in sterile PBS. Resuspend final washed pellet to A_{600} of 0.1

3) Add 10 µl of *B. subtilis* stock to 1 µl of diluent control or diluted antibacterial protein sample in duplicate microcentrifuge tubes. Mix by pipette and incubate at 25° C for 30 min.

   NOTE: 6.2 µg/ml of Penicillin/Streptomycin mix is adequate to inhibit growth of *B. subtilis* for a control.

4) Add 489 sterile TSB to each tube, mix, and aliquot 100 µl into 96-well micro titer plate in quadruplicate. Incubate plate at 25° C (potentially incubate a duplicate plate at 37° C for comparison, assay for loss of activity at higher temp) until control wells reach an $A_{600}$of 0.08 to 0.120. RECORD DATA.

Appendix 3. Procedure for antibiotic screening of cultured libraries (Mote Marine Laboratory):

Bacteria are plated from libraries onto rectangular single well plates containing GASWA, Marine Agar, or other appropriate media. At least 2-3 days growth is required at room temperature to grow organisms to sufficient sized "colonies" for assays. The night before antibiotic assays are to be performed, cultures of tester strains are started. Overnight growth is done at 37ºC with gentle agitation.

On the day of (or the day before) the assays, rectangular plates with cultured libraries are UV-irradiated to kill the colonies being tested. This eliminates cross-contamination when overlaying with the tester strains.

0.8% agar overlays (LB, TSB, GASW and marine broth overlays) for tester strains are prepared, autoclaved, and placed in an incubator at 42ºC with gentle agitation.

Place plates (without lid) in the hood and turn on the UV lamp. Irradiate on high for 15-30 minutes (UV resistance may vary depending on the source of bacteria used in library generation). Mark the plates that are irradiated in some way with a marker to indicate they have been "killed."

1. Assay Set Up:
    a) Remove appropriate agar from incubator
    b) Inoculate with appropriate amount of batch culture (amounts may vary depending on growth stage).

2. Using glass pipette transfer 10ml of 0.8% agar from container to one well plate, move back and forth along center or empty edge of plate dispensing agar, then tilt the plate to distribute, ensure complete covering. Have appropriate tubes, UV irradiated plates, and pipettes warm in the 42ºC incubator.

3. Incubate library plates overnight at 30º C.

4. Identify zones of inhibition and note width from edge of colony using calipers. Record data for each strain in Excel file using frozen storage library grid. Data entered include tester strain active against and diameter in mm of zone of inhibition for each tester strain noted.

Appendix 4. Procedure for antibiotic screening of cultured libraries (University of South Florida Center for Biological Defense)

All isolates were subbed onto marine agar (MA), tryptic soy agar supplemented with 5% sheep red blood cells (BA), thiosulfate citrate bile salts sucrose agar (TCBS), and plain tryptic soy agar (TSA) and incubated for 2 days or longer (up to 5 days) until growth was evident. Growth was quantified and described. Gram stains were made using a P-swab to transfer bacteria to glass slides. Each specimen was methanol-fixed and left to dry. Slides were stained with crystal violet for 1 minute, rinsed with water, saturated with iodine for 1 minute, rinsed with water, de-stained with ethanol for 3 seconds, rinsed with water, and counterstained with safranin for 1 minute. In addition, KOH testing was also performed by placing a loopful of bacterial growth into a drop of 3% potassium hydroxide on a glass slide and mixed thoroughly for 60 seconds. A positive KOH reaction (signifying a Gram-negative bacterium) was demonstrated by the mixture becoming viscous and when the loop was lifted, the mixture formed a string extending from the slide to the loop.

Depending upon the Gram-stain/ KOH reaction, either MEP plates or API biochemical kits were used to further characterize the isolates. API panels were set up per manufacturer's instructions and incubated at 30º C for 24-48 hours (until 3 positive tests occurred). We did not expect to identify the gram negative isolates but wanted more information for each of them. We also wanted to rule out any *Vibrio* isolates since these came from a marine environment. MEP plates were used to help classify the Gram positive bacteria as *Bacillus* spp or not, or members of the *Bacillus cereus* group. Optimal temperatures (25, 30 or 35ºC) were determined for all isolates. Isolates were streaked onto marine agar slants for immediate use and for later use, suspended in marine broth supplemented with 25% glycerol and frozen at -80℃.

For antimicrobial screening, the marine isolates were grown on marine agar for two days at room temperature. Suspensions of these were made in 1mL of marine broth and adjusted to equal a 2 McFarland standard. Then 100 µL of each suspension was placed into a well of a microtiter plate. Using a 48 prong frogger, the bacterial suspensions were inoculated onto marine agar media plates. Orientation of each plate was noted by use of a crystal violet spot in one or two of the wells. Plates were left upright (media side down) for one hour at room temperature, then turned over and left to incubate for 48 hours. In the meantime, testing strains were grown on BA media and checked for purity and subbed so testing would use 24 hour growth.

On testing day, the marine agar plates with the marine isolate spots were placed into the biosafety cabinet media side down and the lids taken off. The growth was exposed to UV light for 45 minutes and the plates were then closed and ready for use. One to five colonies of the testing strains were used to inoculate demineralized water and adjusted to match a 1 McFarland standard. Then 100 µL of that suspension was placed into 20 mL of melted (and cooled to 55-60℃) marine agar and slowly mixed by inversion two times. The agar/bacteria mixture was slowly poured over the marine agar plates containing the dead marine isolate spots and allowed to cover evenly and cooled.

When the plates were cooled and the agar solidified, another layer of uninoculated tryptic soy agar was poured over the agar/bacteria layer and again, the plates were allowed to cool. After 45 minutes, the plates were inverted and incubated at 30℃ overnight.

The next day, the plates were examined for zones of inhibition of growth of the testing strains. The zone diameters were measured. No activity of the marine isolate products against the testing strain was. Activity was described as any zone of no growth above and around the marine isolate colony spot.

Appendix 5. Meeting abstracts

1) Gordon Conference: Oceans and Human Health. University of New England. Biddeford, ME. June 3-8, 2012.

Epidermal mucus from cownose ray (*Rhinoptera bonasus*) and Atlantic stingray (*Dasyatis sabina*): Antimicrobial properties and role in wound healing. Carl Luer, Cathy Walsh, Kim Ritchie, Jodi Miedema, Jennifer Yordy, A.B. Bodine, Andrew Cannons, Vicki Luna, Laura Edsberg, Jen Wyffels

Sharks, and their skate and ray relatives, have been observed to heal rapidly and without infection from wounds obtained naturally in their habitat. A protective secretion produced by epidermal mucus cells in stingrays is being investigated 1) to identify mucus-associated antimicrobial compounds with the potential for development into novel therapeutics to treat wound infection pathogens, and 2) to determine the role of epidermal secretions in wound healing processes. Freshly collected epidermal mucus from cownose rays (*Rhinoptera bonasus*), and Atlantic stingrays (*Dasyatis sabina*) contains at least 20 proteins/protein subunits, based on SDS polyacrylamide gel electrophoresis of an aqueous supernatant obtained through centrifugation. Chemical extraction of fresh mucus with 1) Tris-EDTA, 2) acetic acid and solid phase extraction, and 3) surfactants (Triton X-100, Tween-80, and N-octylglucoside) results in partial purification of mucus compounds. Bacterial symbionts that are not seawater contaminants are found in freshly collected epidermal mucus, and many purified isolates of these symbionts demonstrate antimicrobial activity against tester strains. Forty-six of 384 bacterial isolates cultured from *R. bonasus* and 49 of 227 isolates from *D. sabina* epidermal mucus demonstrated antibiotic activity against at least one human pathogenic test strain in primary screens performed at Mote Marine Laboratory. Of the 46 *R. bonasus* isolates, 13 demonstrated antimicrobial activity against a different panel of pathogenic bacterial tester strains when screened at University of South Florida Center for Biological Defense. Using the GenBank database, BLAST searches performed on 16S rDNA sequence data identified six different genera among 22 of the 49 *D. sabina*-derived bacterial isolates that produced antibacterial compounds against various tester strains. Some active isolates not genetically confirmed through the database may represent undescribed organisms. Preliminary experimental wounding studies with *D. sabina* are in progress, with 1.5 cm diameter circular wounds through epidermal and dermal layers of the skin on dorsal wing surfaces. Wound healing is being documented photographically at weekly intervals, and epidermal mucus on and around the wound is being collected for calculation of healing trajectories and proteomic analysis.

2) Annual Meeting of the American Elasmobranch Society. University of British Columbia, Vancouver, British Columbia, Canada, August 8-14, 2012.

Antimicrobial Properties of Epidermal Mucus from Two Species of Ray (Cownose Ray, *Rhinoptera bonasus*, and Atlantic Stingray, *Dasyatis sabina*). Carl Luer, Cathy Walsh, Kim Ritchie, Jennifer Yordy, Jodi Miedema, ,A.B. Bodine, Andrew Cannons, Vicki Luna

The protective secretion produced by epidermal mucus cells in stingrays is being investigated to identify mucus-associated antimicrobial compounds with the potential for development into novel therapeutics to treat wound infection pathogens. Freshly obtained epidermal mucus from cownose rays (*Rhinoptera bonasus*), and Atlantic stingrays (*Dasyatis sabina*) can be separated by gentle centrifugation into an aqueous supernatant and a viscous pellet. The aqueous supernatant contains at least 20 proteins/protein subunits based on SDS polyacrylamide gel electrophoresis. Chemical extraction of fresh mucus with 1) Tris-EDTA, 2) acetic acid and solid phase extraction, and 3) selected surfactants (Triton X-100, Tween 80, and N-octylglucoside) results in partial purification of mucus compounds. Fresh mucus also contains bacterial symbionts that are not seawater contaminants. Forty-six of 384 bacterial isolates cultured from *R. bonasus* and 49 of 227 isolates from *D. sabina* epidermal mucus demonstrated antibiotic activity against at least one human pathogenic tester strain in primary screens performed at Mote Marine Laboratory. Of the 46 *R. bonasus* isolates, 13 demonstrated antimicrobial activity against a different panel of pathogenic bacterial tester strains when screened at University of South Florida Center for Biological Defense. Using the GenBank database, BLAST searches performed on 16S rDNA sequence data identified six different genera among 22 of the 49 *D. sabina*-derived bacterial isolates that produced antibacterial compounds against various tester strains. Some of the active isolates not genetically confirmed with the database could be undescribed organisms. Culturable libraries of all isolates have been cryopreserved.